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Development of a Multilocus Sequence Typing Tool for High-Resolution Genotyping of *Enterocytozoon bieneusi*▽

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Thus far, genotyping of *Enterocytozoon bieneusi* has been based solely on DNA sequence analysis of the internal transcribed spacer (ITS) of the rRNA gene. Both host-adapted and zoonotic (human-pathogenic) genotypes of *E. bieneusi* have been identified. In this study, we searched for microsatellite and minisatellite sequences in the whole-genome sequence database of *E. bieneusi* isolate H348. Seven potential targets (MS1 to MS7) were identified. Testing of the seven targets by PCR using two human-pathogenic *E. bieneusi* genotypes (A and Peru10) led to the selection of four targets (MS1, MS3, MS4, and MS7). Further analysis of the four loci with an additional 24 specimens of both host-adapted and zoonotic *E. bieneusi* genotypes indicated that most host-adapted genotypes were not amplified by PCR targeting these loci. In contrast, 10 or 11 of the 13 specimens of the zoonotic genotypes were amplified by PCR at each locus. Altogether, 12, 8, 7, and 11 genotypes of were identified at MS1, MS3, MS4, and MS7, respectively. Phylogenetic analysis of the nucleotide sequences obtained produced a genetic relationship that was similar to the one at the ITS locus, with the formation of a large group of zoonotic genotypes that included most *E. bieneusi* genotypes in humans. Thus, a multilocus sequence typing tool was developed for high-resolution genotyping of *E. bieneusi*. Data obtained in the study should also have implications for understanding the taxonomy of *Enterocytozoon* spp., the public health significance of *E. bieneusi* in animals, and the sources of human *E. bieneusi* infections.

Of the 14 or so human-pathogenic microsporidia species, *Enterocytozoon bieneusi* is the most common, causing chronic diarrhea in AIDS patients and acute diarrhea in immunocompetent persons (4). In addition to causing human disease, *E. bieneusi* is frequently found in many animals, especially mammals (9). Thus, microsporidiosis by *E. bieneusi* is a potential zoonotic disease. Indeed, zoonotic transmission of *E. bieneusi* infection from guinea pigs to a child has been reported (3). Nevertheless, little is known about the transmission routes of *E. bieneusi* in humans and domestic animals and the significance of zoonotic infection in microsporidiosis epidemiology.

DNA sequencing tools based on the internal transcribed spacer (ITS) of the rRNA gene have been used widely in genotyping *E. bieneusi* infecting humans and animals (8). These studies have identified the presence of host-adapted *E. bieneusi* genotypes in various domestic animals and wild mammals, as well as a large group of *E. bieneusi* genotypes that do not appear to have any host specificity (5, 11–13). The latter genotypes without host specificity are considered zoonotic and are responsible for most human infections. In human infections, *E. bieneusi* genotypes have been shown to differ from each other in geographic distribution (5) and virulence (2). It remains to be determined whether these observations are also true for other genetic loci.

In this study, we screened the *E. bieneusi* genome for microsatellite and minisatellite sequences and developed a multilocus sequence typing (MLST) technique for high-resolution typing of parasites from humans and various animals.

MATERIALS AND METHODS

Specimens. A total of 26 *E. bieneusi* specimens were used in the study, including 13 (each) of the zoonotic and host-adapted genotypes as determined by ITS sequence analysis. The specimens of zoonotic genotypes came from two children, seven HIV-positive (HIV^+) adults, three pigs, and one chicken in Brazil and Peru (Table 1), whereas those of the host-adapted genotypes came from three raccoons, two (each) cattle, dogs, and muskrats, and one (each) goat, cat, guinea pig, and marmoset in the United States, Portugal, and Peru (Table 2). Most of the specimens were genotyped in previous studies (3, 7, 10–12), whereas the remaining specimens were genotyped by the same technique and included in this study (Tables 1 and 2). DNA preparations from two specimens of zoonotic genotypes (specimen identification codes 6562 and 6653) were used in the initial evaluation of PCR targets. The remaining DNA preparations were used in PCR analyses of the targets that were eventually chosen.

Microsatellite and minisatellite identification. An initial search for microsatellite and minisatellite sequences in the three large scaffolds (GenBank accession numbers NW_001849444 to NW_001849446) generated in the *E. bieneusi* Whole

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TABLE 1. Specimens of zoonotic *E. bieneusi* genotypes used in the study[a]

ID	Host	Source location	ITS genotype (GenBank accession no.)	PCR product size (bp) (GenBank accession no.)				Reference(s)
				MS1	MS3	MS4	MS7	
4343	HIV$^+$	Brazil	WL12 (JF927952)	655 (JF951429)	515 (HQ615895)	885 (HQ615909)	465 (HQ615925)	This study
4424	HIV$^+$	Brazil	NIA1 (JF927953)	−	511 (HQ615897)	704 (HQ615910)	462 (HQ615916)	This study
4447	HIV$^+$	Brazil	D (JF927954)	−	−	−	465 (HQ615924)	This study
6975	HIV$^+$	Peru	Type IV (AY371277)	613 (JF951428)	515 (HQ615892)	+	462 (HQ615922)	2, 10
7925	HIV$^+$	Peru	Peru11 (AY371286)	637 (JF951430)	515 (HQ615896)	+	465 (HQ615923)	2, 10
6562	HIV$^+$	Peru	A (AY371276)	616 (HQ615886)	511 (HQ615898)	+	463 (HQ615914)	2, 10
6653	HIV$^+$	Peru	Peru10 (AY371285)	652 (HQ615889)	515 (HQ615893)	885 (HQ615907)	462 (HQ615920)	2, 10
10204	Child	Peru	Peru15 (EF014431)	+	−	−	−	3
6539	Child	Peru	Peru6 (JF927955)	670 (HQ615887)	−	793 (HQ615906)	450 (HQ615912)	This study
5436	Pig	Peru	EbpC (JF927956)	+	509 (HQ615899)	887 (HQ615904)	462 (HQ615918)	This study
7119	Pig	Peru	EbpC (JF927957)	640 and 637 (HQ615890 and HQ615891)	509 (HQ615901)	−	−	This study
7141	Pig	Peru	EbpC (JF927958)	637 (HQ615888)	509 (HQ615900)	887 (HQ615905)	462 (HQ615919)	This study
13153	Chicken	Peru	Peru8 (JF927959)	+	513 (HQ615894)	885 (HQ615908)	462 (HQ615921)	This study

[a] Shown are the amplification efficiencies of specimens of zoonotic *E. bieneusi* genotypes at the four selected microsatellite and minisatellite loci. ID, specimen identification code; HIV$^+$, HIV-positive adult; +, PCR positive but produced noisy signals at sequencing; −, PCR negative.

Genome Shotgun Project (GenBank accession number ABGB00000000) was conducted on 12 June 2008. A secondary search of all 1,743 contigs (ABGB01000001 to ABGB01001743) from the project was conducted on 21 August 2009. Microsatellite and minisatellite sequences were defined as sequences with tandem repeats of ≤6 and >6 nucleotides, respectively. They were identified in the retrieved sequences using the software Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html).

PCR analysis of microsatellite and minisatellite targets. A nested PCR was used in the amplification of microsatellite and minisatellite targets. For each locus, the primary and secondary PCR primers were designed based on nucleotide sequences flanking the potential microsatellite and minisatellite repeats. The potential targets were amplified by nested PCR, using 1 μl of DNA in the primary PCR and 2 μl of primary PCR products in the secondary PCR. For both the primary and secondary PCR, the PCR mixture consisted of 200 mM (each) deoxynucleotide triphosphates, 1× PCR buffer (Applied Biosystems, Foster City, CA), 3.0 mM $MgCl_2$, 5.0 U of *Taq* polymerase (Promega, Madison, WI), and 100 nM primers in a total volume of 100 μl. The reactions were performed with a GeneAmp PCR 9700 thermocycler (Applied Biosystems) for 35 cycles at 94°C for 45 s, at the annealing temperature specified in Table 3 for 45 s, and at 72°C for 60 s, with an initial denaturation (94°C for 5 min) and a final extension (72°C for 10 min). To neutralize PCR inhibitors, 400 ng/μl of nonacetylated bovine serum albumin (Sigma-Aldrich, St. Louis, MO) was used in the primary PCR. The secondary PCR products were detected by agarose gel electrophoresis and ethidium bromide staining. Each DNA was analyzed by PCR at least twice, and both PCR products were sequenced.

Sequence analysis. The secondary PCR products were sequenced in both directions with an ABI 3130 genetic analyzer (Applied Biosystems) using the secondary primers and a BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems). The sequences obtained were aligned with each other using ClustalX (http://www.clustal.org/). To assess the genetic relatedness of

TABLE 2. Specimens of host-adapted *E. bieneusi* genotypes used in the study[a]

ID	Host	Source location	ITS genotype (GenBank accession no.)	PCR product size (bp) (GenBank accession no.)				Reference
				MS1	MS3	MS4	MS7	
9175	Marmoset	Portugal	Marmoset genotype PtEb XII (DQ885588)	607 (HQ615883)	−	897 (HQ615911)	459 (HQ615917)	7
10216	Guinea pig	Peru	Guinea pig genotype Peru16 (EF014427)	+	529 (HQ615903)	−	459 (HQ615915)	3
5999	Muskrat	United States	Muskrat genotype WL5 (AY237213)	607 (HQ615885)	527 (HQ615902)	+	456 (HQ615913)	11
3548	Muskrat	United States	Muskrat genotype WL4 (AY237212)	−	−	−	−	11
9171	Cat	Portugal	Muskrat genotype PtEb VIII (DQ885584)	−	−	−	−	7
3603	Raccoon	United States	Raccoon genotype WL3 (AY237211)	−	−	−	−	11
3609	Raccoon	United States	Raccoon genotype WL2 (AY237210)	−	−	−	−	11
3624	Raccoon	United States	Raccoon genotype WL1 (AY237209)	598 (HQ615884)	−	−	−	11
9256	Dog	Portugal	Dog genotype PtEb IX (DQ885585)	+	−	−	−	7
2640	Dog	United States	Dog genotype PtEb IX (DQ885585)	−	−	−	−	This study
9269	Cattle	Portugal	Bovine genotype PtEb XI (DQ885587)	−	−	−	−	7
6164	Cattle	United States	Bovine genotype J (AY331005)	−	−	−	−	12
16887	Goat	Peru	Bovine genotype EBB6 (EU153584)	−	−	−	−	This study

[a] Shown are the amplification efficiencies of specimens of host-adapted *E. bieneusi* genotypes at the four selected microsatellite and minisatellite loci. ID, specimen identification code; +, PCR positive but produced noisy signals at sequencing; −, PCR negative.

TABLE 3. Primer sequences of microsatellite and minisatellite loci selected for evaluation

Locus	GenBank accession no. (nucleotide positions)	Locus tag and description[a]	Targeted repeat[b]	Primers[c]	Annealing temp (°C)	Expected product size (bp)
MS-1	ABGB01000003 (63854–64529)	EBI_22576, tRNA methyltransferase	$(TAT)_{31}$, $(TAG)_{11}$	F1, CAA GTT GCA AGT TCA GTG TTT GAA	58	843
				R1, GAT GAA TAT GCA TCC ATT GAT GTT		
				F2, TTG TAA ATC GAC CAA ATG TGC TAT	58	676
				R2, GGA CAT AAA CCA CTA ATT AAT GTA AC		
MS-2[d]	ABGB01001554 (367–787)	EBI_27022, hypothetical protein (MS in the intergenic region)	$(TG)_{19}$	F1, GTA CAA GAT GAA GTT CCT GAG T	55	584
				R1, CAT GAC ATC ATT TTA CAT ACA CAT		
				F2, GGC CTG ATA ATA GAT CGG ATT	55	421
				R2, CAG CAT CAT CAC ACG TTC TCA		
MS-3	ABGB01000035 (202–738)	EBI_27665, hypothetical protein (MS in the intergenic region)	$(TA)_{21}$	F1, CAA GCA CTG TGG TTA CTG TT	55	702
				R1, AAG TTA GGG CAT TTA ATA AAA TTA		
				F2, GTT CAA GTA ATT GAT ACC AGT CT	55	537
				R2, CTC ATT GAA TCT AAA TGT GTA TAA		
MS-4	ABGB01000033 (1063–1947)	EBI_21785, translation initiation factor 2 (MS in the coding region), intergenic region; EBI_25436, LSU ribosomal protein L22P	(TTATTTTTTCCATTTTTCTTCTTCTATTTCCTTTA)$_9$	F1, GCA TAT CGT CTC ATA GGA ACA	55	965
				R1, GTT CAT GGT TAT TAA TTC CAG AA		
				F2, CGA AGT GTA CTA CAT GTC TCT	55	885
				R2, GGA CTT TAA TAA GTT ACC TAT AGT		
MS-5[d]	ABGB01000169 (546–1144)	EBI_23156, ATPase component of ABC transporter	(GCGGCTGGTTTCGCAGCAGCGGTTTTAGCAACTGGCTTC)$_{12}$	F1, GTC ATG ATC ACC GGC ACT TA	55	882
				R1, CTC AAG GAT CGT CAA GCT GA		
				F2, GCA GGC TTT GCA GTT GGC TT	55	599
				R2, GTG AAG GAA GCC GTA GCT AA		
MS-6[d]	ABGB01000562 (660–1157)	Not annotated	$(AT)_{14}$	F1, GAA TAG AAT GAT TCT AGC CAT GA	55	706
				R1, CCA TAT AGC CTT TAA GAC CAA A		
				F2, CTT TTC AAG GAT GGT TTG AAT GA	55	498
				R2, CAA AGG GTA CCT CCA ATC AAA		
MS-7	ABGB01000014 (23807–24277)	EBI_27441 (23,137–24,093) and EBI_27442 (24144–25052), hypothetical proteins (MS is in EBI_27441)	$(TAA)_{13}$	F1, GTT GAT CGT CCA GAT GGA ATT	55	684
				R1, GAC TAT CAG TAT TAC TGA TTA TAT		
				F2, CAA TAG TAA AGG AAG ATG GTC A	55	471
				R2, CGT CGC TTT GTT TCA TAA TCT T		

[a] MS, minisatellite or microsatellite.
[b] Targeted repeat, tandem repeat identified in the sequence of *E. bieneusi* in the whole-genome sequencing project.
[c] F, forward; R, reverse.
[d] Did not produce the expected PCR products in the initial primer evaluations.

various *E. bieneusi* genotypes, neighbor-joining trees were constructed using the program TreeconW (http://bioinformatics.psb.ugent.be/software/details/3), based on the evolutionary distances calculated by the Kimura two-parameter model.

Nucleotide sequence accession numbers. Unique sequences generated in this study have been deposited in the GenBank database under accession numbers HQ615883 to HQ615925, JF927952 to JF927959, and JF951428 to JF951430.

RESULTS

Identification of microsatellite and minisatellite sequences in the *E. bieneusi* genome. The initial search for tandem repeats in the three large scaffolds in the *E. bieneusi* genome sequencing project (GenBank accession numbers NW_001849444,

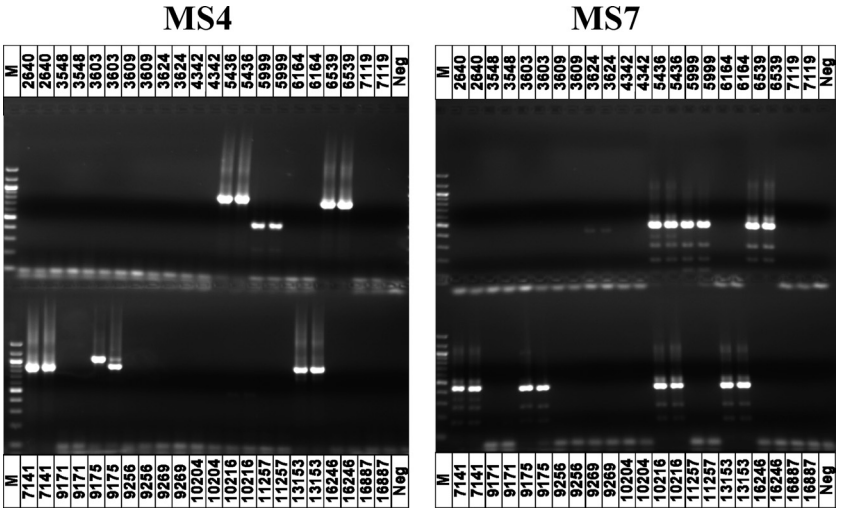

FIG. 1. PCR amplification of *E. bieneusi* specimens at MS4 (left panel) and MS7 (right panel). Most host-adapted ITS genotypes (2640, 3548, 3603, 3609, 3624, 5999, 6164, 9171, 9175, 9256, 9269, 10216, and 16887) were not amplified at either locus.

NW_001849445, and NW_001849446) identified the presence of 9, 16, and 4 targets, respectively. All the targets with tandem repeats have three or fewer copies, with the exception of two microsatellite sequences (TAT and TAG repeats) in NW_001849445, which were located adjacent to each other and had 31 and 11 copies of the repeat sequences, respectively. This locus was selected and designated MS1.

A second search of all 1,743 contigs (GenBank accession numbers ABGB01000001 to ABGB01001743) from the genome sequencing project identified the presence of microsatellite and minisatellite sequences in 113 contigs. Based on the nature (imperfect repeats being largely absent) and length (>6 copies for minisatellite targets and >10 copies for microsatellite targets) of the repeats and the availability of suitable sequences for primer design (excluding those with short or AT-rich 5′ or 3′ flanking nucleotide sequences), six additional potential targets (MS2 to MS7) on different contigs were chosen from the 113 sequences, including four microsatellite loci and two minisatellite loci (Table 3). The location of the loci was not considered, as the *E. bieneusi* genome was not fully assembled and annotated at the execution of the study.

Primers for nested PCR were designed for the seven loci, with expected PCR products ranging from 421 to 885 bp (Table 3).

PCR analysis of the selected genetic loci. Testing of the seven potential targets by PCR analysis of two human-pathogenic *E. bieneusi* genotypes (A and Peru10) led to the selection of four targets (MS1, MS3, MS4, and MS7). The other three loci generated products of the wrong size (MS2) or no products at all (MS5 and MS6). Further analysis of the four loci with an additional 11 specimens of the zoonotic ITS genotypes of *E. bieneusi* led to the generation of PCR products around the expected size in 8 or 9 specimens (Table 1), although there were some obvious differences in the sizes of MS4 products (Fig. 1). These four loci were also analyzed with 13 specimens of the host-adapted *E. bieneusi* genotypes. Only 5, 2, 2, and 3 of them generated the expected PCR products at the MS1, MS3, MS4, and MS7 loci, respectively (Table 2). For most of the specimens, PCR products of the same size were generated in duplicate analyses of each specimen at each locus. However, at MS4, specimen 9175 had PCR products of two sizes in the duplicate analyses (Fig. 1), and specimens 6975 and 7925 each generated three bands in all PCR replicates (data not shown).

DNA sequence analysis of MS1, MS3, MS4, and MS7. Positive PCR products of the amplified loci were sequenced successfully, with the exception of 5 and 4 specimens at the MS1 and MS4 loci, respectively, which produced unreadable sequences with numerous underlying signals in the electropherogram. For specimen 9175, only the smaller of the two MS4 products yielded a readable sequence. For most of the specimens, two sequences obtained at each locus were identical to each other, with the exception of specimen 7119, which produced two sequences that differed from each other in the copy number (13 versus 14 copies) of the TAA repeat (Fig. 2). Altogether, 11, 12, 8, and 14 specimens were sequenced successfully at the MS1, MS3, MS4, and MS7 loci, representing 12 (one specimen had concurrent infection with two genotypes), 8, 7, and 11 genotypes of *E. bieneusi*, respectively. For each locus, the sequences generated from specimens of the zoonotic ITS genotypes differed from each other mostly in the number of microsatellite and minisatellite repeats, although a few single nucleotide polymorphisms (SNPs) were seen in 5′ and 3′ nonrepeat regions. The sequences obtained from the few specimens of the host-adapted ITS genotypes at MS1, MS4, and MS7, however, differed from each other significantly and had numerous SNPs compared to those from the zoonotic genotypes (Fig. 3). At MS3, the sequence differences between the two groups of ITS genotypes were similar to those within each group (data not shown).

Genetic relationship among *E. bieneusi* genotypes. A neighbor-joining tree was constructed with sequences obtained at each locus, with the most divergent sequence as the out-group. Results of the analysis support the conclusion made from the direct sequence comparisons, with the sequences from host-adapted ITS genotypes of *E. bieneusi* placed at the base of the tree and those from the zoonotic ITS genotypes forming a

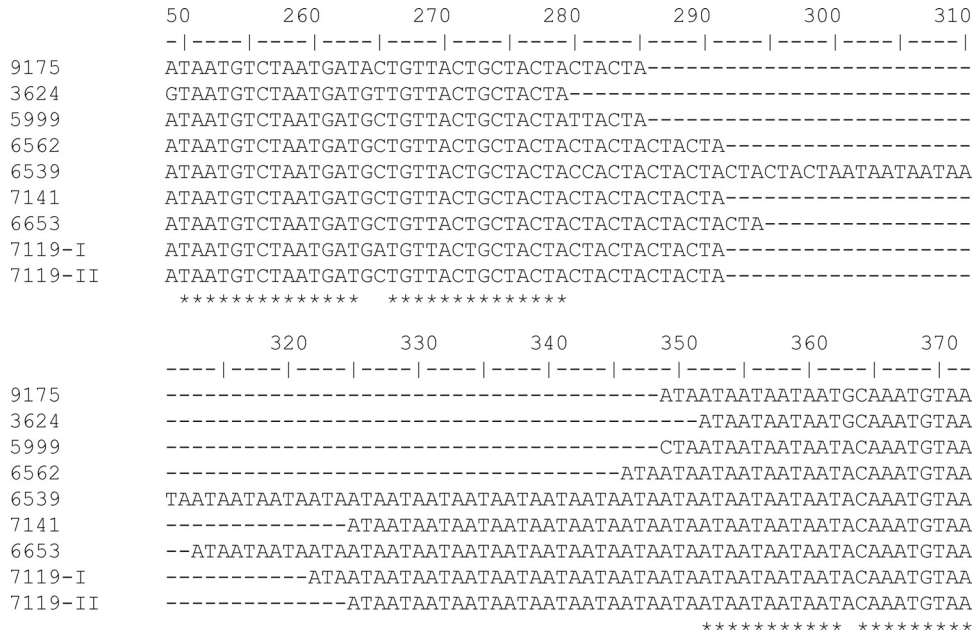

FIG. 2. Variations in the number of microsatellite repeats of MS-1 among *E. bieneusi* specimens. Columns with nucleotides identical to those of the marmoset genotype PtEb XII (specimen 9175) are indicated by asterisks at the bottom of the sequence alignment. Specimen 3624 is the ITS raccoon genotype WL1. The remaining sequences are zoonotic genotypes.

large cluster. The only exception was MS3, which did not have a monophyletic cluster containing all sequences from specimens of the zoonotic ITS genotypes (Fig. 4).

DISCUSSION

Microsatellite and minisatellite sequences are common targets in the development of genotyping tools for the differentiation of eukaryotic organisms. They have been used successfully in genotyping two human-pathogenic microsporidian parasites, *Encephalitozoon cuniculi* and *Encephalitozoon hellem* (14, 15). In this study, we took advantage of the recent genome sequence surveys of *E. bieneusi* (1, 6) and identified four microsatellite and minisatellite markers that showed high sequence polymorphism in the *E. bieneusi* specimens examined. Together with the ITS, they became part of an MLST tool for genotyping *E. bieneusi* in humans and animals.

Data generated from this study support the existence of two large groups of *E. bieneusi*: zoonotic genotypes and host-adapted genotypes. Thus, primers based on sequences from the genome sequence surveys of the human isolate H348

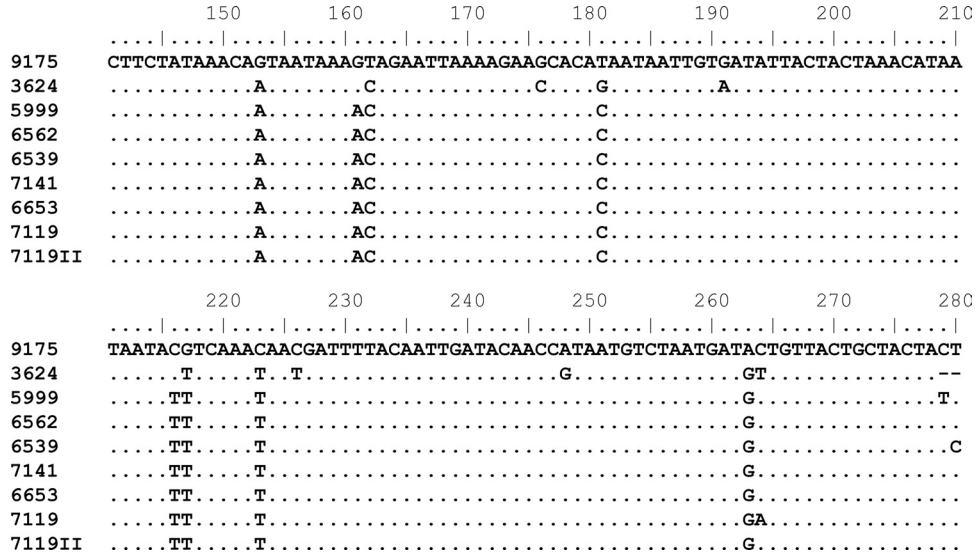

FIG. 3. Sequence variation in part of the nonrepeat region of MS-1 among *E. bieneusi* specimens. Dots denote nucleotide identity to that of the marmoset genotype PtEb XII (specimen 9175). Specimen 3624 is the ITS raccoon genotype WL1. The remaining sequences are zoonotic genotypes.

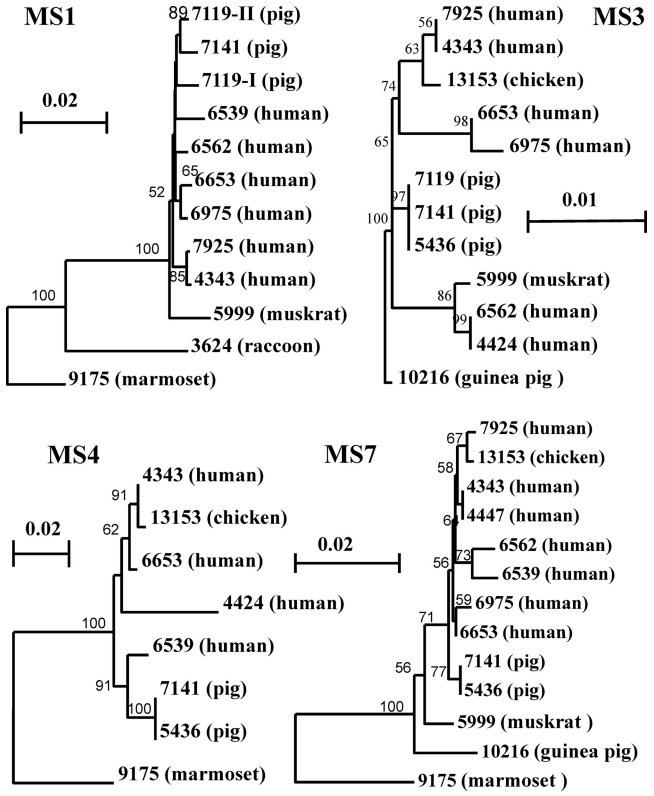

FIG. 4. Phylogenetic relationship among genotypes of *E. bieneusi* at four microsatellite and minisatellite loci (MS1, MS3, MS4, and MS7) as assessed by a neighbor-joining analysis of the nucleotide sequences, using distance calculated by the Kimura two-parameter model. The host origins of the sequences are specified in parentheses.

largely failed to amplify DNA from host-adapted ITS genotypes of *E. bieneusi*, and the few sequences obtained from this group had nucleotide sequences very divergent from those obtained from the zoonotic ITS genotypes. This observation was further supported by results of the phylogenetic analyses of most loci, with sequences from zoonotic genotypes forming one large cluster and sequences from host-adapted genotypes placed at the base of the trees. The only exception was MS3, which produced sequences largely similar to each other. Even with MS3, DNA of most host-adapted genotypes failed to be amplified by PCR, and we could not be sure that the few sequences from the host-adapted genotypes were not from zoonotic genotypes that coinfected the animals.

Interestingly, one of the targets, MS4, generated PCR products of two different sizes in a replicate analysis of specimen 9175 from a marmoset (Fig. 1). Two other specimens (6975 and 7925) from HIV^+ adults in Peru each generated three PCR products in all PCR analyses. The exact reason for this is not clear, as the larger PCR product of 9175 and all products from 6975 and 7925 produced noisy sequences. The MS4 sequences generated in this study were analyzed by BLAST using MicrosporidiaDB (http://microsporidiadb.org/), which identified three contigs from the genome sequence survey project that had high sequence similarities (>95%): ABGB01000033 (contig 1043), ABGB01000014 (contig 678), and ABGB01000963 (contig 394). The first two contigs covered the full MS4 target, whereas contig 394 terminated before the minisatellite repeats. In ABGB01000033, MS4 spans nucleotide positions 1063 to 1947 and is located at the 3′ end of the locus EBI_21785 and 5′ end of EBI_25436, which complement nucleotide positions 1272 to 2489 and 654 to 1175 of ABGB01000033, respectively. In contrast, in ABGB01000014, MS4 spans nucleotide positions 128590 to 129267 and is located at the 3′ end of the locus EBI_27505 (positions 128280 to 129071 of ABGB01000014) and the intergenic region after the locus. At both loci, the minisatellite region of MS4 is located in the coding region of EBI_25436 and EBI_27505, unlike those in MS2 and MS3, which are located in the intergenic region. Although contigs ABGB01000033 and ABGB01000014 had similar nucleotide sequences in the nonrepeat region of MS4, the MS4 sequence in ABGB01000014 is much shorter and had a total of 144 bp deletions in the minisatellite region. In addition, ABGB01000033 and ABGB01000014 had very different sequences flanking the MS4 target, and ABGB01000014 had a 23-bp deletion in the F2 primer region, including the last 8 bp of the primer. Thus, there could be three copies of the MS4 target in the *E. bieneusi* genotype, an idea which was supported by PCR analysis of DNA from specimens 6975 and 7925. Although some *E. bieneusi* isolates had divergent sequences for each copy of the targets, most other *E. bieneusi* isolates in this study probably had identical MS4 sequences among the copies. Previously, 19 protein-coding genes in the *E. bieneusi* genome were identified as having multiple copies (1), although MS4 was not among them.

In conclusion, four polymorphic microsatellite and minisatellite markers were identified for genetic characterizations of *E. bieneusi*, and an MLST tool for genotyping *E. bieneusi* was developed. The tool should be useful in epidemiologic investigations of *E. bieneusi* transmission, especially those concerning the public health significance of parasites of animal origin. It should also be useful in the revision of *Enterocytozoon* taxonomy and the characterization of the population genetics of the parasite, especially the potential role of either host species or geography in genetic structuring. These studies should analyze a larger number of specimens from more diverse regions and assess the relationship among MLST genotypes, host specificity, virulence or clinical presentations, and risk factors.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

REFERENCES

1. **Akiyoshi, D. E., et al.** 2009. Genomic survey of the non-cultivatable opportunistic human pathogen, *Enterocytozoon bieneusi*. PLoS Pathog. **5:**e1000261.
2. **Bern, C., et al.** 2005. The epidemiology of intestinal microsporidiosis in patients with HIV/AIDS in Lima, Peru. J. Infect. Dis. **191:**1658–1664.
3. **Cama, V. A., et al.** 2007. Transmission of *Enterocytozoon bieneusi* between a child and guinea pigs. J. Clin. Microbiol. **45:**2708–2710.
4. **Ghosh, K., and L. M. Weiss.** 2009. Molecular diagnostic tests for microsporidia. Interdiscip. Perspect. Infect. Dis. **2009:**926521.
5. **Henriques-Gil, N., M. Haro, F. Izquierdo, S. Fenoy, and C. del Aguila.** 2010. Phylogenetic approach to the variability of the microsporidian *Enterocyto-*

zoon bieneusi and its implications for inter- and intrahost transmission. Appl. Environ. Microbiol. **76:**3333–3342.
6. **Keeling, P. J., et al.** 2010. The reduced genome of the parasitic microsporidian *Enterocytozoon bieneusi* lacks genes for core carbon metabolism. Genome Biol. Evol. **2:**304–309.
7. **Lobo, M. L., et al.** 2006. Genotypes of *Enterocytozoon bieneusi* in mammals in Portugal. J. Eukaryot. Microbiol. **53**(Suppl. 1)**:**S61–S64.
8. **Santin, M., and R. Fayer.** 2009. *Enterocytozoon bieneusi* genotype nomenclature based on the internal transcribed spacer sequence: a consensus. J. Eukaryot. Microbiol. **56:**34–38.
9. **Santin, M., and R. Fayer.** 2011. Microsporidiosis: *Enterocytozoon bieneusi* in domesticated and wild animals. Res. Vet. Sci. **90:**363–371.
10. **Sulaiman, I. M., et al.** 2003. A molecular biologic study of *Enterocytozoon bieneusi* in HIV-infected patients in Lima, Peru. J. Eukaryot Microbiol. **50**(Suppl)**:**591–596.
11. **Sulaiman, I. M., et al.** 2003. Molecular characterization of microsporidia indicates that wild mammals harbor host-adapted *Enterocytozoon* spp. as well as human-pathogenic *Enterocytozoon bieneusi*. Appl. Environ. Microbiol. **69:**4495–4501.
12. **Sulaiman, I. M., et al.** 2004. Molecular characterization of *Enterocytozoon bieneusi* in cattle indicates that only some isolates have zoonotic potential. Parasitol. Res. **92:**328–334.
13. **Widmer, G., and D. E. Akiyoshi.** 2010. Host-specific segregation of ribosomal nucleotide sequence diversity in the microsporidian *Enterocytozoon bieneusi*. Infect. Genet. Evol. **10:**122–128.
14. **Xiao, L., et al.** 2001. Genotyping *Encephalitozoon hellem* isolates by analysis of the polar tube protein gene. J. Clin. Microbiol. **39:**2191–2196.
15. **Xiao, L., et al.** 2001. Genotyping *Encephalitozoon cuniculi* by multilocus analyses of genes with repetitive sequences. J. Clin. Microbiol. **39:**2248–2253.